Distribution of Plasmid-Borne Stress Protein Genes in *Streptococcus thermophilus* and Other Lactic Acid Bacteria

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Abstract. The presence of heat stress protein genes (*hsp*) was tested by Southern hybridization analysis in total DNA extracts from species of the genus *Streptococcus* (47 strains), *Lactobacillus* (34 strains), *Lactococcus* (24 strains), and *Leuconostoc* (5 strains). The biotinylated *hsp16.4* probe prepared from an ORF2 fragment of pER341 (2.8 kb) tested positively with restricted DNA extracts of seven *Streptococcus thermophilus* strains and a single strain of *Lactococcus lactis* subsp. *cremoris*. In all positive *S. thermophilus* strains, the *hsp* was located on plasmids ranging from ca. 2.8 kb to 11 kb in size, while *hsp* was present in a 7.5-kb plasmid in *Lactococcus lactis* subsp. *cremoris*. Southern blots with a *rep* probe showed that all *hsp16.4*$^+$ plasmids in *S. thermophilus* strains also shared homology with the replication function (*rep*) of pER341, suggesting the common origin of these plasmids.

Lactic acid bacteria (LAB) constitute a heterogeneous group of microbes that include members of the genus *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc*. Many species of LAB are used in combination as essential biocatalytic agents in the production of fermented dairy foods, to carry out biochemical reactions under a variety of controlled conditions that result in hundreds of different fermented dairy foods.

LAB cultures are subjected to stressful conditions during preproduction, production, and postproduction phases of dairy food fermentations. Stress may be induced by lyophilization or concentration, extreme temperatures (heat, cold), salt treatment (NaCl), rising lactic acid concentration with concomitant pH shift, and nutrient deprivation. These and possibly other types of stress induced by production parameters may impact on the survival of LAB cultures and the performance of biochemical activities at a desired level. In recent years, interest has grown in the stress response phenomenon of LAB species, since understanding the mechanism of stress response may lead to the development of cultures with improved capacity to survive and function under industrial production conditions. Studies have included heat stress response in *Lactococcus lactis* subsp. *lactis* [4], *Lactobacillus delbrueckii* subsp. *bulgaricus* [18], and *Streptococcus thermophilus* [3, 16], salt stress response in *Lactococcus* lactis subsp. *lactis* [10], and lactic acid stress response in lactococci [11] and *S. thermophilus* [6]. Heat, acid, and salt stress, as a rule, elicit in LAB cultures an increased production of specific, chromosomally encoded, high-molecular-weight class proteins known as heat stress proteins or Hsps [2, 3, 5, 7, 10]. However, a 17-kDa class Hsp was also found overexpressed in heat-stressed *L. lactis* subsp. *lactis* [2], and overproduction of a family of low-molecular-weight (approximately 16 kDa) Hsps was also observed in acid-stressed *S. thermophilus* [6]. Our laboratory recently reported the increased synthesis of a 142-amino acid plasmid-borne heat stress protein (Hsp16.4), when *S. thermophilus* ST134 was subjected to heat stress [16]. The association of Hsps with plasmids in LAB cultures is especially interesting as it may offer a convenient way for introducing Hsp genes (*hsp*) to different hosts, leading to a possible increase either in specific or multiple stress tolerance in recipient LAB cultures of industrial importance.

We report the distribution of *hsp* genes for low-molecular-weight Hsps in *S. thermophilus* and other LAB cultures based on Southern hybridization analysis of

restricted total DNA extracts and plasmids. The probe was a 258-bp fragment of *hsp16.4*, which encodes Hsp16.4 and is carried by the 2798-bp pER341, a native plasmid of *S. thermophilus* ST134. DNA preparations were also examined for shared homology with the replication gene (*rep*) of pER8, a related 2-kb cryptic plasmid of *S. thermophilus* ST108.

Materials and Methods

Bacterial strains, media, and growth conditions. Several strains of *Lactococcus lactis* subsp. *lactis* (21), *L. lactis* subsp. *cremoris* (3), *Lactobacillus acidophilus* (4), *L. delbrueckii* subsp. *bulgaricus* (4), *L. casei* subsp. *casei* (3), and 42 strains of *Streptococcus thermophilus* were from our laboratory collection. Other species of *Lactobacillus* (23) and *Leuconostoc* (5) were obtained from the National Center for Agricultural Utilization Research culture collection (Peoria, IL), whereas species of *Streptococcus* (5) were from the American Type Culture Collection (Manassas, VA). Lactococci and *S. thermophilus* strains were maintained in tryptone–yeast extract–lactose (TYL) medium [13] at 30°C and 37°C, respectively. Lactobacilli and leuconostocs were grown in MRS medium (Difco Laboratories, Detroit, MI), while streptococci were grown in brain heart infusion medium (Difco) at 30°C or 37°C, as required.

DNA isolation and manipulations. Plasmid DNA was extracted from cells by a standard protocol [14], adapted to 35 ml of broth culture of each test microorganism. Total DNA was extracted from 100 ml of broth cultures by a modification of the same protocol to prevent denaturation and elimination of chromosomal DNA. High-purity plasmids were prepared by CsCl-gradient ultracentrifugation [17] or by the Elutip-d protocol (Schleicher & Schuell, Inc., Keene, NH). Restriction endonucleases for DNA fragmentation were purchased from BRL Life Technologies (Gaithersburg, MD) and used under conditions recommended by the supplier. All total DNA extracts were digested with *Eco*RI. Plasmid DNAs were digested with single- or multisite restriction endonucleases selected after a preliminary survey of plasmids for digestibility with a battery of enzymes. *Eco*RI digests of total DNA were analyzed by agarose (0.7%) gel electrophoresis (AGE) in TBE buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M Na-EDTA, pH 8.3), at 10 V for 16 h. Samples of restricted plasmids were resolved in 1.2% agarose gels at 125 V for 2 h. DNA bands were detected in ethidium bromide-stained gels by transmitted UV illumination.

Preparation of biotinylated probes. The purified native plasmid pER341 (2.8 kb) of *S. thermophilus* ST134 was digested with *Hin*fI, and fragments generated were resolved by AGE. The 258-bp fragment corresponding to coordinates 2014 and 1756 within the open reading frame (ORF2) of the heat stress protein gene (*hsp16.4*, nt# 2,029-1,601) in pER341 [16] was excised, recovered from gel slices with GenElute Agarose Spin Columns (Supelco, Inc., Bellefonte, PA), and purified with Elutip-d columns. Biotinylation of the fragment was done by nick translation with the BioNick Labeling System (BRL Life Technologies).

To establish relatedness among the replication functions of plasmids investigated, a biotinylated probe was also prepared from the 522-bp *Bst*EII/*Hin*dIII fragment of pER8 (2.0 kb) from *S. thermophilus* ST108. The fragment represents approximately 55% of the replication function (ORF1 or *rep*) in this cryptic plasmid, which was previously shown to share a high degree of homology with the ORF1 (*rep*) of pER341 [16].

Southern hybridization analysis. For detecting DNA sequence homologies, fragments generated by restricting total DNA samples with *Eco*RI were resolved by AGE, depurinated, denatured, and vacuum transferred to nylon membranes in an Oncor ProbeTech I automated Southern blot system (Oncor, Inc., Gaithersburg, MD), according to the manufacturer's recommended procedures. Hybridization of digested total DNA extracts with the biotinylated *hsp16.4* probe was carried out at 45% formamide concentration for 18 h at 42°C. Posthybridization steps including washes, filter blocking, streptavidin and alkaline phosphatase treatment, and staining with nitrotetrazolium blue and 5-bromo-4-chloro-3-indolyl phosphate was also performed according to the supplier's suggested protocol (Oncor, Inc.). Plasmid samples were prepared for hybridization analysis by the same procedures outlined above, following the digestion of each plasmid with a specific restriction endonuclease, and resolving linearized plasmids or plasmid fragments generated by AGE.

When applicable, sequence comparisons of Hsp16.4 with other putative Hsps were done with the aid of the BLASTP database program [1].

Results

Hybridization analysis with total DNA digests. Southern blot hybridization with the biotinylated *hsp16.4* probe was first carried out with total DNA extracts from all test cultures, including streptococci, lactococci, leuconostocs, and lactobacilli. Of the 110 total DNA extracts tested, only eight samples reacted positively with the biotinylated *hsp16.4* probe. This group included DNA digests from seven strains of *S. thermophilus*, and *L. lactis* subsp. *cremoris* ATCC 14365. The results of Southern blots with total DNA extracts generally confirmed that low-molecular-weight, heat-stress proteins sharing homology with 16-kDa class Hsps reported earlier in *S. thermophilus* [6, 16] and, by inference, with other related and chromosomally encoded low-MW, heat-stress proteins produced by *Clostridium acetobutylicum* [12] and *Leuconostoc oenos* [9] are not widely distributed among lactic acid bacteria and are found primarily in various strains of *S. thermophilus*.

Southern blots prepared with the biotinylated *rep* probe from pER8 showed a positive response with digests of 12 total DNA preparations, all isolated from *S. thermophilus* strains, including the 7 *hsp16.4*$^+$ extracts. On the basis of the data obtained with the *hsp16.4* and *rep* probes, further work was focused on strains of *S. thermophilus* and the single, *hsp16.4*$^+$ strain of *L. lactis* subsp. *cremoris* ATCC 14365.

Hybridization analysis with plasmid DNA digests. *S. thermophilus* strains (12) and *L. lactis* subsp. *cremoris* ATCC 14365 with total DNA extracts reacting positively with either of the biotin-labeled *hsp16.4* and *rep* probes were analyzed for the presence of plasmid DNA. All 12 *S. thermophilus* cultures and *L. lactis* subsp. *cremoris* ATCC 14365 were found to carry plasmid DNA. Most *S. thermophilus* carried a single plasmid, confirming earlier findings [14], while *L. lactis* subsp. *cremoris* ATCC 14365 contained at least five plasmids. Purified

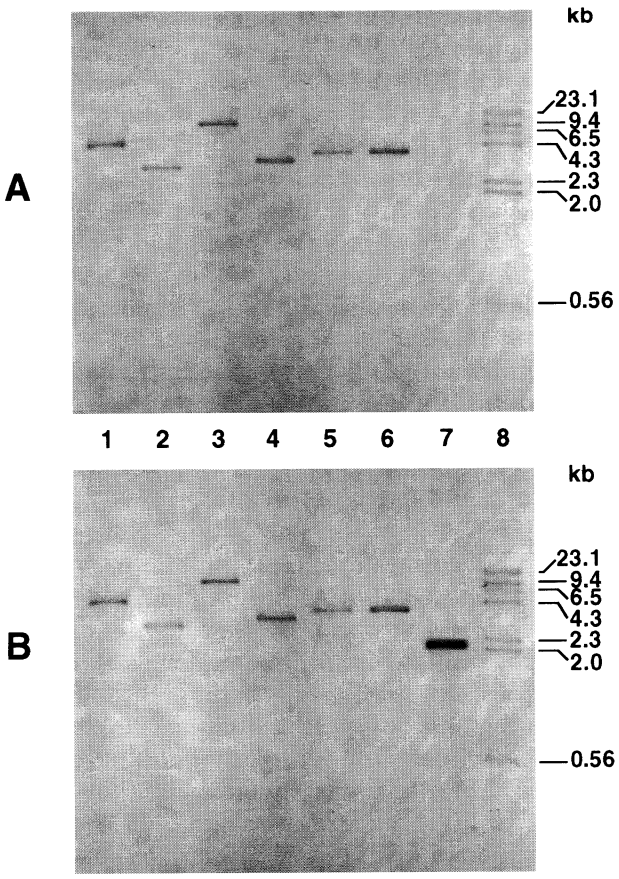

Fig. 1. Southern hybridization reactions of *S. thermophilus* plasmid digests with biotin-labeled *hsp16.4* (A) and *rep* (B) probes. Lanes: 1, pER16/*Hpa*I; 2, pER341/*Hae*III (positive control for *hsp16.4*); 3, pER35/*Bst*EII; 4, pER35/*Hin*dIII; 5, pER36/*Hha*I; 6, pER41/*Hae*III; 7, pER8/*Hin*dIII (positive control for *rep*); 8, λ/*Hin*dIII MW markers. (C) Southern blot of plasmid digests from *L. lactis* subsp. *cremoris* ATCC 14365 with biotinylated *hsp16.4* probe. Lanes: 1, *Hae*III digest; 2, *Hha*I digest; 3, λ/*Hin*dIII MW markers.

plasmid preparations from these strains were prepared for Southern blot analysis by digestion with a restriction enzyme that was selected to yield the least number of DNA fragments in order to limit scattering of hybridization signals. The target plasmids were tested for hybridization with the biotinylated *hsp16.4* and *rep* probes. The range of test plasmids was expanded by including as negative controls plasmid preparations from five additional *S. thermophilus* strains whose total DNA extracts failed to give a positive signal earlier with either biotinylated probe.

The results of Southern hybridizations are summarized in Fig. 1. The pattern of plasmid DNA hybridizations with the *hsp16.4* (Fig. 1A) and *rep* (Fig. 1B) probes provided presumptive evidence that several *S. thermophilus* plasmids of the same size may be identical. Since the 2-kb size group included plasmids pER1, pER8, pER19, pER20, and pER27, and the 4.5-kb size plasmid group included pER7, pER16, and pER26, Fig. 1A and 1B show hybridization signals with only one representative member of each of the two plasmid groups. Southern blots identified seven plasmids, three of which may be identical, that gave strong signals with both *hsp16.4* and *rep* probes. Since all *S. thermophilus* plasmids were linearized with specific, single-site restriction endonucleases, hybridization with biotin-labeled *hsp16.4* or *rep*, as expected, showed only a single band which corresponded to the molecular mass of the plasmid. However, at least in the case of the 11-kb pER35, which was digested to several fragments by *Hin*dIII, both *hsp16.4*$^+$ and *rep*$^+$ signals were located on an approximately 3.7-kb fragment. Plasmids in the 2-kb size group reacted only with the *rep* probe, and five other *S. thermophilus* plasmids failed to hybridize with either probe. Plasmid digests of the only other reactive member of the LAB group, *L. lactis* subsp. *cremoris* ATCC 14365, probed positively with biotinylated *hsp16.4* but not with *rep* (Fig. 1C). The *hsp16.4*$^+$ signal in this digest was associated with an approximately 7.5-kb plasmid designated as pLLC75, and digestion with *Hha*I pinpointed its location on an approximately 4.7-kb fragment. The hybridization signal with pLLC75 DNA required prolonged development, which provided putative evidence of a lower degree of homology of this plasmid-borne *hsp* with the *hsp16.4* of pER341 in *S. thermophilus* ST134.

As shown in Table 1, the positively responding LAB plasmids, predominantly from *S. thermophilus*, may be arranged in four groups on the basis of hybridization

Table 1. Hybridization analysis of LAB plasmids with *hsp16.4* and *rep* probes

Group	Source	Plasmid	Size (kb)	Hybridization *hsp16.4*	Signal with *rep*
I	*S. thermophilus*	pER7	4.5	+	+
		pER16	4.5	+	+
		pER26	4.5	+	+
		pER341	2.8	+	+
		pER35	11.0	+	+
		pER36	3.7	+	+
		pER41	3.6	+	+
II	*S. thermophilus*	pER1	2.09	−	+
		pER8	2.09	−	+
		pER19	2.09	−	+
		pER20	2.09	−	+
		pER27	2.09	−	+
III	*S. thermophilus*	pER13	4.3	−	−
		pER342	9.5	−	−
		pER371	2.7	−	−
		pER372	9.7	−	−
		pER43	6.5	−	−
IV	*L. lactis* subsp. *cremoris*	pLLC75	7.5	+	−

tests. The first group of seven plasmids, ranging from approximately 2.8 to 11 kb in size and including five major plasmid types, hybridized with both *hsp16.4* and *rep* probes. Best estimates of a molecular mass of approximate 4.5 kb and restriction mapping data indicated that three plasmids in this group (pER7, pER16, and pER26) may be one and the same plasmid species. The second group includes five plasmids with identical size (approximately 2 kb), which hybridized with only the *rep* probe but not with the *hsp16.4* probe. Maps generated by restriction endonuclease analysis for plasmids found in this group indicated that most likely they are the same plasmid species. The third group included *S. thermophilus* plasmids that failed to hybridize with either probe. Plasmids in this group ranged from approximately 2.7 to 9.5 kb in size. The only member of the fourth group was the 7.5-kb pLLC75 from *L. lactis*subsp. *cremoris* ATCC 14365, which signaled homology with the *hsp16.4* but not with the *rep* probe.

Discussion

Dairy fermentation bacteria, similar to other prokaryotic systems, respond to various environmental stresses (heat, acid, salt) by synthesizing stress proteins, including low-MW (<30 kDa) Hsps. Detection of stress-induced production of low-MW Hsps has been limited to lactococci [2] and *S. thermophilus* [6, 16]. Since information on the occurrence of low-MW Hsps in lactic acid bacteria would be valuable in elucidating the possible physiological functions of these proteins, several species of representative genera of the LAB group were surveyed for the presence of low-MW Hsps, with the biotinylated fragment of *hsp16.4* as the probe.

Our Southern hybridization studies revealed several important aspects about the dynamics of distribution of low-MW Hsp 16.4-like stress proteins in lactic acid bacteria. Although homology of the plasmid-borne Hsp16.4 of *S. thermophilus* ST134 chromosomally controlled 18-kDa class Hsps from prokaryotic (*C. acetobutylicum*, *L. oenos*) and eukaryotic (plants) sources was demonstrated [16], data collected in this study indicated that stress protein genes homologous to *hsp16.4* occur primarily in *S. thermophilus* strains and, with the exception of *L. lactis* subsp. *cremoris* ATCC 14365, rarely in other LAB cultures. We also concluded that in all *hsp16.4*$^+$ ST cultures examined, and also in *L. lactis* subsp. *cremoris* ATCC 14365, homologous *hsp* genes are located on plasmids and, at least in the case of *S. thermophilus*, the five *hsp16.4*$^+$ plasmids also share homologous replication (*rep*) domains. By inference, these findings may be applicable to other *S. thermophilus* plasmids that were not available for this study. For example, although the data presented earlier by others allowed one to conclude the association of an *hsp* with a 2.77 kb plasmid (pST1) in *S. thermophilus*, owing to sequencing errors the identity of *hsp* was not recognized [8]. Nevertheless, sequence comparison of Hsp16.4 with the nontranslated putative ORF_{hsp} of pST1 shows 83% homology between the two Hsps. Although unpublished, sequence data on other putative low-MW Hsps in a 6.5-kb plasmid (pCI65st) of *S. thermophilus* NDI-6 also may be retrieved from the GenBank database (accession #AF027167, deposited by T.F. O'Sullivan and G.F. Fitzgerald). Sequence comparisons indicated 80% and 58% homology between Hsp16.4 and the putative plasmid-borne Hsp1 (150 amino acids) and Hsp2 (102 amino acids), respectively, in this *S. thermophilus* strain. Another report describing the overexpression of a 16-kDa protein family consisting of five members and varying from 16.5 kDa to 16.9 kDa in acid-stressed *S. thermophilus* PB18 showed relatedness of two Hsps (approximately 16.7 kDa and 16.9 kDa) to the putative Hsp encoded on pST1 [6]. However, this report lacks evidence for the association of any of the low-MW Hsps with the 3.5-kb plasmid of strain PB18, although by analogy our data strongly suggest that at least one of the Hsps may be encoded on this plasmid.

Previous research on homology among *S. thermophilus* plasmids, which was carried out with biotin-labeled fragments of pER8 as probes, already provided putative evidence for the common evolutionary origin of this

group of *S. thermophilus* plasmids [15]. The current study, relying on more exacting hybridization tests with biotin-labeled fragments of *rep* from pER8 and *hsp16.4* from pER341 as probes, confirmed the accuracy of the earlier homology grouping of ST plasmids. It is possible to hypothesize that the five *S. thermophilus* plasmids identified as carriers of homologous *hsp16.4* and *rep* genes (Table 1) may have evolved from a common ancestral plasmid (e.g., pER8) which at one point acquired a prokaryotic *hsp* responsible for the production of a low-MW stress protein. In addition, in isolated cases, plasmids of other LAB cultures with unrelated replication domains (*rep*), such as the lactococcal pLLC75, may also have acquired *hsp* genes that share homology with *hsp16.4* present in pER341.

Although an auxiliary role has been suggested for low-MW Hsps in protecting *Lactococcus* cells from environmental stress [2], the precise physiological functions of stress-induced low-MW Hsps in LAB cultures still await elucidation. The availability of plasmids carrying *hsp* genes provides an important tool to accelerate studies on the mechanism and control of *hsp* expression in lactic acid bacteria. Plasmid-borne *hsp* genes will also facilitate the transfer of *hsp* genes into industrial strains of LAB cultures, possibly leading to improved survival and enhanced performance under production conditions.

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